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Comparison and optimization of methods for the simultaneous extraction of DNA, RNA, proteins, and metabolites

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ABSTRACT

The challenge of performing a time-resolved comprehensive analysis of molecular systems has led to the quest to optimize extraction methods. When the size of a biological sample is limited, there is demand for the simultaneous extraction of molecules representing the four areas of "omics": genomics, transcriptomics, proteomics, and metabolomics. Here we optimized a protocol for the simultaneous extraction of DNA, RNA, proteins, and metabolites and compared it with two existing protocols. Our optimization comprised the addition of a methanol/chloroform metabolite purification before the separation of DNA/RNA and proteins. Extracted DNA, RNA, proteins, and metabolites were quantitatively and/or qualitatively analyzed. Of the three methods, only the newly developed protocol yielded all biomolecule classes of adequate quantity and quality.

Systems biology as an interdisciplinary field is focused on the understanding of cellular systems at the molecular level. Various "omic" technologies are used to gain insight into the complex interactions of biomolecules within biological systems on a comprehensive scale [1]. Technical developments in genomics, transcriptomics, proteomics, and metabolomics enable monitoring and quantification of biomolecules in a high-throughput manner [2–4]. The integration of omic technologies is difficult when the same sample is not used for comprehensive molecular analysis. Hence, it is necessary to develop methods for the simultaneous extraction and effective recovery of the target biomolecules DNA, RNA, proteins, and metabolites. So far, protocols exist for isolating three of the four molecule classes [5,6] or all four from prokaryotes [7], but there is currently no reliable method for the simultaneous extraction of the four molecule classes from eukaryotic cells.

The goal of this study was to optimize a method for the simultaneous extraction of DNA, RNA, proteins, and metabolites and compare it with two existing, well-established methods that were designed to extract only one class of molecules specifically. We applied it to the analysis of Jurkat T cells as a model for native human T cells [8], and in order to rule out cell-line-specific effects we also tested the process by analyzing murine hepatocyte cells.

Our method (here method C) was based on our good experience with the simultaneous purification of RNA, DNA, and proteins starting with a phenol/chloroform-based extraction. To also purify metabolites, we added a methanol/chloroform-based extraction of metabolites prior to the phenol/chloroform-based steps.

Our method was compared with that (here method A) reported by Weckwerth and coworkers describing the concomitant extraction of RNA, proteins, and metabolites from plant material [9]. We subsequently probed the quality of the remaining DNA fractionated by this protocol. In addition, a third method (here method B), based on the manufacturer's protocol of the TRI Reagent for RNA, DNA, and protein isolation with an additional step included to extract metabolites, was tested (http://www.sigma-aldrich.com) for the two different cell lines.

The suitability of the extraction methods for the biomolecule classes was evaluated by assessing the quantity and quality of DNA

Abbreviations used: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; RT, room temperature; gDNA, genomic DNA; PCR, polymerase chain reaction; RIN, RNA integrity number; qPCR, quantitative PCR; LC, liquid chromatography; MS/MS, tandem mass spectrometry; IC, ion chromatography; MRM, multiple reaction monitoring; GRAVY, grand average of hydropathy; MW, molecular weight.

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and RNA, the number of proteins and proteome coverage, and the detection of hydrophilic metabolites of the central carbon and nitrogen metabolism. Because the protocols tested resulted in differing extraction efficacies for the omic technologies, this study will aid in selecting the most suitable method for a specific research question.

Materials and methods

Cell culture

Human Jurkat T cells (clone E6-1, ATCC, Germany) were cultured in RPMI-1640 medium containing 10% (v/v) fetal calf serum, 1% (v/v) streptomycin (100 mg/ml)/penicillin (100 U/ml), and 1% (v/v) L-glutamine in a CO_2 incubator (MCO-18AIC, Sanyo Electric, Japan) at 37 °C and an atmosphere of 5% CO_2. The cells were cultured at a density of 1×10^6 cells/ml. Murine Hepa 1c1c7 cells (clone CRL-2026, ATCC, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 1% (v/v) streptomycin (100 mg/ml)/penicillin (100 U/ml), and 1% (v/v) L-glutamine in a CO_2 incubator at a density of 1×10^4 cells/cm^2. Cell viability and cell numbers were determined using trypan blue and a Neubauer-improved chamber (Optic Labor, Germany). Aliquots of 1×10^7 cells were washed twice with 1× phosphate-buffered saline (PBS) and centrifuged at 300 *g* for 5 min. The obtained cell pellets were resuspended in 50 µl of PBS and processed via method A, B, or C or well-established protocols. Each method was conducted in triplicate.

Method A

The pelleted Jurkat T or Hepa cells were processed as described by Weckwerth and coworkers [9] with modification as follows. The RNA buffer phase was directly subjected to acetic acid/ethanol precipitation of nucleic acids. An extraction buffer of 0.05 M Tris (pH 7.6), 0.5% sodium dodecyl sulfate (SDS), and 1% β-mercaptoethanol was used. The precipitated nucleic acids were resuspended in 100 µl of water and proteins in 300 µl of 1 M urea and 0.05 M Tris (pH 7.6) for further analysis. DNA and RNA samples were stored at −80 °C, and metabolite and protein samples were stored at −20 °C, until further analysis.

Method B

To a pellet of Jurkat T or Hepa cells, 50 µl of 1× PBS and 1 ml of TRI Reagent (Sigma–Aldrich Chemie, Steinheim, Germany) were added and processed according to the manufacturer's instructions with modifications as follows. After TRI Reagent/chloroform extraction, RNA was precipitated by the addition of 0.5 ml ice-cold isopropanol and incubation for 10 min at room temperature, followed by centrifugation for 10 min at 4 °C and 4000 *g*. The supernatant containing the metabolites was subsequently transferred to a fresh tube and dried under vacuum. DNA was resuspended in 300 µl of 8 mM NaOH, RNA in 50 µl of H_2O, and proteins in 100 µl of 1% SDS.

Method C

An aliquot of Jurkat T or Hepa cells was dissolved in 1 ml of a 45% (v/v) methanol/5% (v/v) chloroform solution and incubated for 30 min at 4 °C while rotating. The cell suspension was centrifuged at 500 *g* for 10 min, and the metabolite-containing supernatant was transferred to a new tube and dried under vacuum.

The remaining pellet was resuspended in 100 µl of 1× PBS. Approximately 1 ml of H_2O saturated phenol was added for separation of RNA, DNA, and proteins, and the sample was incubated for 5 min at 4 °C while rotating. For phase separation, 200 µl of chloroform was added, and the sample was incubated for 5 min at room temperature (RT), followed by centrifugation at 12,000 *g* for 15 min at 4 °C. After phase separation, the upper RNA-containing phase was mixed with 500 µl of ice-cold isopropanol, incubated for 10 min at RT, and centrifuged for 20 min at 12,000 *g* at 4 °C. The obtained RNA pellet was washed using 75% (v/v) ethanol, air dried for 10–15 min at RT, and resuspended in 50 µl of H_20. To the remaining middle and lower phases of the phenol chloroform extraction, 500 µl of DNA extraction buffer (4 M guanidinium thiocyanate, 50 mM sodium citrate, and 1 M Tris base) was added. The solution was mixed by inversion, incubated for 30 min at RT, and centrifuged for 20 min at 12,000 *g* at 4 °C. For DNA precipitation, 500 µl of ice-cold isopropanol containing 20 µg/ml glycogen was added to the upper and middle phases. After incubation for 10 min at RT, the solution was centrifuged for 20 min at 12,000 *g* at 4 °C. The DNA-containing pellet was washed twice by adding 1.5 ml of 75% (v/v) ethanol and centrifuged for 10 min at 7500 *g*. The pellet was dried at RT for 10 min and resuspended in 100 µl of TE buffer.

For protein extraction, 1.5 ml of ice-cold isopropanol was added to the remaining lower phase, and the sample was incubated overnight at −20 °C to precipitate the proteins. The solution was then centrifuged for 15 min at 12,000 *g* at 4 °C. The protein pellet was washed by the addition of 1 ml 0.3 M guanidinium chloride in 90% (v/v) ethanol, incubation for 10 min at RT, and subsequent centrifugation for 5 min at 7500 *g* at 4 °C. The pellet was washed a second time with 1 ml of ethanol. The protein pellet was dried for 20–30 min at RT and dissolved in 100 µl of 6 M urea/2 M thiourea in 100 mM ammonium bicarbonate buffer.

Well-established control methods

For isolation of DNA from Jurkat and Hepa cells, the genomic DNA (gDNA) isolation kit from Zymo Research (Orange, CA, USA) was used following the manufacturer's instructions. The DNA was eluted from columns in 100 µl of TE buffer.

RNA from Jurkat and Hepa cells was extracted using the Qiagen RNeasy Mini Kit (Hilden, Germany). For elution of RNA from the columns, 50 µl of H_2O was used.

Extraction of Jurkat and Hepa cell proteins was performed by lysing the cells with 6 M urea/2 M thiourea in 100 mM ammonium bicarbonate for 5 min at RT, followed by ultrasonication for 30 s. Samples were subsequently centrifuged for 5 min at 12,000 *g* at 4 °C, and the protein-containing supernatant was transferred to a new tube.

For extraction of metabolites, a hot water method (method GS1) and a boiling ethanol method (method GS2) were conducted as described by Canelas and coworkers [10].

DNA quality control

The quantity of isolated DNA was determined for all methods by an ND spectrophotometer (Thermo Scientific, Germany), and the quality was tested by 0.8% (w/v) agarose gel electrophoresis. A 16.7-µl sample of DNA extracted via method A and 600-ng samples of DNA extracted via methods B and C were each mixed with 6× DNA Loading Dye (Thermo Scientific). Genomic DNA showing a distinct signal in the high molecular range of approximately 20 kB was considered to be intact or only partially degraded at the end of the extraction procedure.

For evaluation of DNA quality, Jurkat DNA samples were further analyzed by polymerase chain reaction (PCR) using the DNA Quality Ready Kit (Bio-Budget Technologies, Krefeld, Germany) following the manufacturer's instructions. PCR batches were calculated on a

total volume of 12 μl. A sample of 48 ng gDNA (methods B, C, and GS) and 2.4 μl of 1:10 diluted gDNA extracted via method A were inserted into the gDNA quality control PCR. PCR products were separated on a 1.8% (w/v) agarose gel. The amplification of six PCR fragments of 100, 200, 300, 400, 500 (control fragment), and 600 bp of gDNA indicates that the corresponding DNA was not found to be degraded after the extraction process. Fewer than six amplicons showed DNA with any degree of degradation.

RNA quality control

The quantity of extracted RNA was determined by an ND spectrophotometer (Thermo Scientific), and its quality was assessed by analyzing 10 μl (method A) or 1.5 μg (methods B, C, and GS) on 1.8% (w/v) agarose gel. Each sample was mixed with 2× RNA Loading Dye (Thermo Scientific), applied to a gel, and run for 45 min at 100 V. RNA samples showing the characteristic ribosomal RNA bands of 5070 and 1869 bp with no background noise were considered to be intact, or only slightly degraded, at the end of the extraction procedure.

Next, 1 μl of 1:3 diluted RNA (method A) and 50 ng of extracted RNA (methods B, C, and GS) were applied to an RNA Nano Chip (Agilent Technologies, Waldbronn, Germany) and run on an Agilent 2100 Bioanalyzer following the manufacturer's instructions. RNA with RNA integrity number (RIN) values greater than 8 were considered to be intact and usable for quantitative PCR (qPCR) and other follow-up experiments.

Protein quality control

The quantity of isolated proteins was determined using Quick Start–Bradford Dye Reagent according to the manufacturer's instructions (Bio-Rad Laboratories, Germany). The quality of extracted proteins was evaluated by separation of 30 μg proteins by SDS–PAGE (polyacrylamide gel electrophoresis), followed by Coomassie Brilliant Blue staining according to the method described in Ref. [11]. The protein lane of each sample was cut into four slices, and each slice (~7.5 μg of protein) was digested with 150 ng of trypsin. Generated peptides were extracted from the gel pieces and analyzed via liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a nano-HPLC (high-performance liquid chromatography) system (nanoAcquity, Waters, Milford, MA, USA) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), and liquid chromatography was carried out using a 110-min gradient with 0.1% formic acid in 100% water (solvent A) and 100% acetonitrile (solvent B). After sample injection into a trapping column (nanoAcquity UPLC [ultra-performance liquid chromatography] column, C18, 180 μm × 20 mm, 5 μm particles, Waters), peptides were separated on a C18 column (nanoAcquity UPLC column, C18, 7 μm × 150 mm, 1.7 μm particles, Waters) using the following gradient of solvent B in solvent A: starting at 2%, reaching 6% after 5 min, 20% after 45 min, 30% after 70 min, 40% after 75 min, 85% after 80 min, and 2% after 95 min and washing for 15 min at 2% with a flow rate of 300 nl/min.

Full-scan MS spectra (from 300 to 2000 m/z, $R = 60{,}000$) were acquired in a positive ion mode in the LTQ–Velos Orbitrap. Up to the 10 most intense ions per scan with a charge greater than 2 were fragmented and analyzed in the linear trap. Peptide ions exceeding an intensity of 3000 were chosen for collision-induced dissociation within the linear ion trap (isolation width = 4 m/z, normalized collision energy = 35%, activation time = 30 ms, activation $q = 0.25$). For MS/MS acquisition, a dynamic exclusion of 2 min was applied. For a more detailed description, see Baumann and coworkers [12]. Data analysis of the MS results was performed using MaxQuant (version 1.3.0.5) including the following search parameters: ion mass tolerance of 0.5 Da and parent ion tolerance of 20 ppm. Carbamidomethylation of cysteine was specified as a fixed modification. Oxidation of methionine and acetylation of the protein N terminus were specified as variable modifications. MaxQuant was set up to search a reverse concatenated database of all human proteins annotated in the SwissProt database (version 10/01/2013) assuming the digestion enzyme trypsin. Only proteins found in two of three replicates were considered to be unambiguously identified.

Metabolite analysis

For ion chromatography–tandem mass spectrometry (IC–MS/MS)-based analysis of metabolites, extracts were dissolved in a total volume of 25 μl and analyzed on an ICS-5000 (Thermo Fisher Scientific, Dreieich, Germany) coupled to an API 5500 QTrap (AB Sciex) as described elsewhere [13]. Separation was obtained on an IonPac AS11-HC column (2 × 250 mm, Thermo Fisher Scientific) with an increasing potassium hydroxide gradient. MS analysis was performed in multiple reaction monitoring (MRM) mode using negative electrospray ionization and included organic acids, carbohydrates, and nucleotides involved in central metabolite pathways. Metabolites were considered to be detectable above a signal-to-noise ratio of 3 within a retention time window of 0.5 min.

Results and discussion

Time and material required for performing the three extraction methods

The optimized protocols used to separate the target biomolecules from a single sample of Jurkat T cells or Hepa cells is shown in Fig. 1; the others are shown in the online supplementary material (Figs. S1 and S2).

Method A was based on a study describing the simultaneous extraction of RNA, proteins, and metabolites from plant material [9]. Metabolites are extracted by a methanol/chloroform treatment, leaving a pellet that can be used for further protein, DNA, and RNA extraction using phenol-based phase separation [9]. Notably, this protocol does not produce separation of RNA from DNA.

Method B was based on the TRI Reagent manufacturer's protocol (Sigma–Aldrich) for RNA, DNA, and protein isolation. The cell pellet was resuspended in TRI Reagent, enabling the successive isolation of RNA, DNA, and proteins. Metabolites were obtained from the supernatant remaining after precipitation of RNA, and proteins were obtained by vacuum drying of the respective fraction.

Method C involved a novel process of methanol/chloroform-based extraction of metabolites followed by RNA, DNA, and protein extraction using a phenol/chloroform extraction.

The three methods required similar time and materials. Protocol A could be performed in 4 h plus an additional hour the following day for centrifugation and ethanol washing to remove remaining phenol from proteins isopropanol-precipitated overnight. Using method B, it was possible to extract all four biomolecule classes in 4 h because proteins were not precipitated overnight. Protocol C could be carried out in 3 h plus an additional 1.5 h the following day for pelleting and phenol depletion of proteins after overnight precipitation. Each method contained a step of phenol/chloroform-based phase partitioning, enabling the separation of nucleic acids from proteins. Protocols A and C involved methanol/chloroform-based metabolite extraction steps prior to DNA, RNA, and protein isolation. Considering only time and material required, method B would be the protocol of choice, the disadvantage being that only

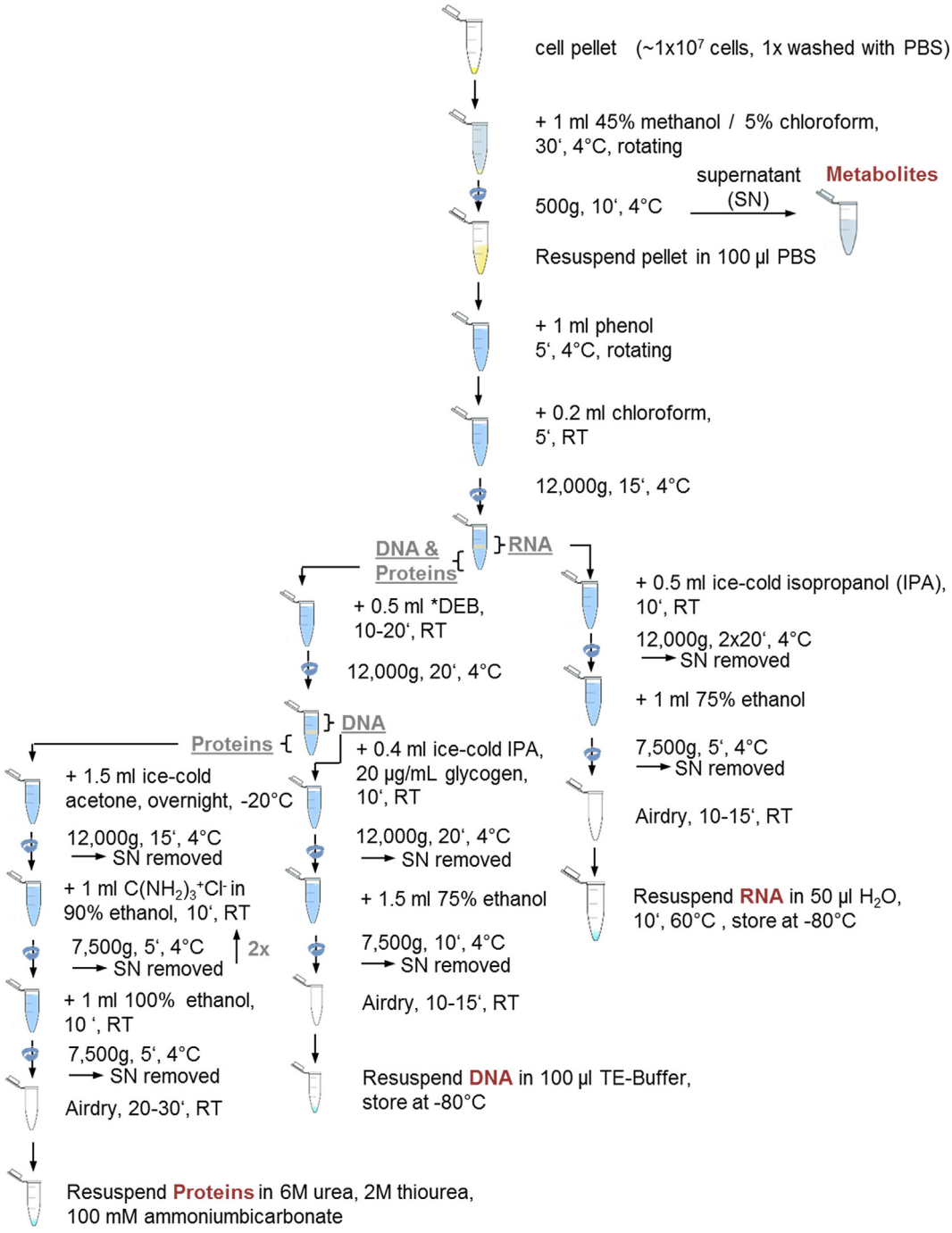

Fig.1. Schematic of extraction procedure. The newly developed process for the extraction of four biomolecule classes from a single set of cells is depicted. *DEB, DNA extraction buffer (method C; see Supplemental figure).

three of the four molecule classes—namely DNA, RNA, and proteins—can be isolated.

DNA isolation: enrichment strategies, purity, and yield

DNA samples from method A also contained RNA when separated on an agarose gel because no further partitioning of the nucleic acid classes was conducted. In addition, DNA extracted via method A was found to be partially degraded at the end of the purification on the agarose gel. With methods B and C and the well-established method (GS), the extracted DNA did not show RNA content as judged by agarose gel or signs of degradation in Jurkat or Hepa cells (data not shown). For assessing quality and integrity, the extracted Jurkat DNA was further analyzed by PCR using the DNA Quality Ready Kit (Bio-Budget Technologies). PCR products were analyzed by agarose gel electrophoresis. DNA extracted via method A showed weaker signal intensities for the expected PCR products than that derived via methods B, C, and GS (Fig. 2A). The kit is designed for human samples, and application to murine Hepa cell DNA was unsuccessful. Methods B and C can be used for the extraction of gDNA, showing acceptable DNA integrity and purity for further applications.

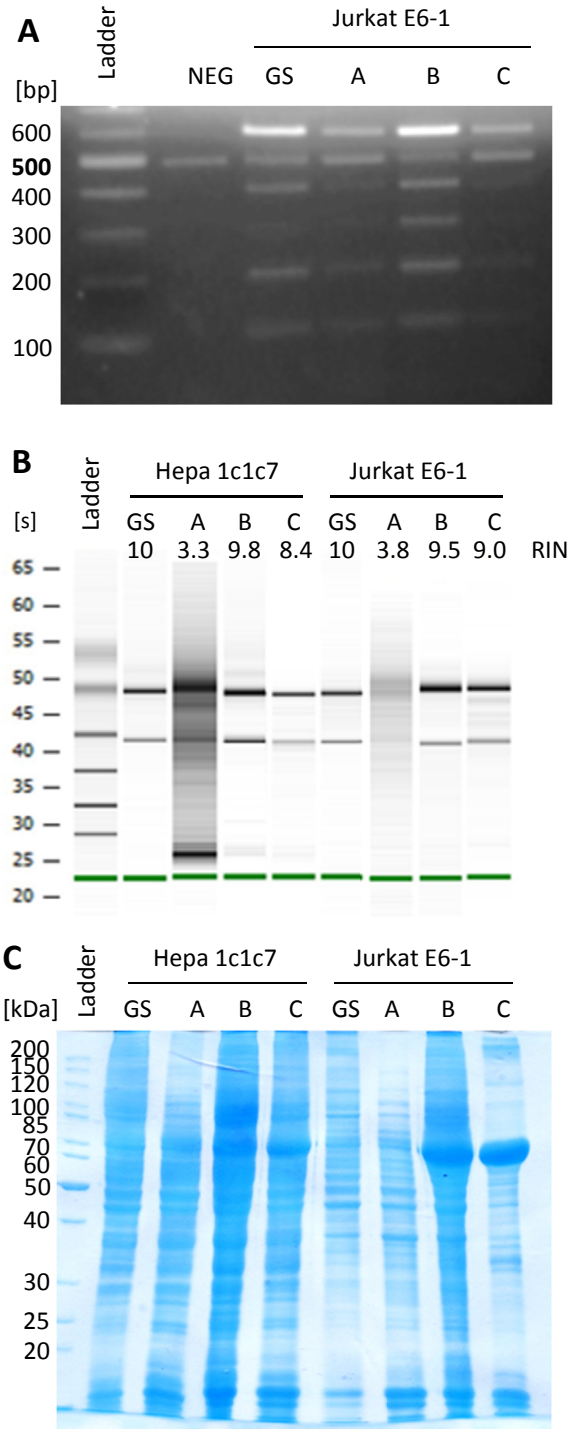

Fig.2. Quality assessment of three of the four molecule classes isolated. (A) PCR for gDNA quality determination. Here 48 ng of gDNA (methods B and C) and 1 μl of 1:10 diluted gDNA extracted via method A were inserted into gDNA quality control PCR. (B) Results of Agilent 2100 Bioanalyzer measurements. Here 50 ng of RNA (methods B and C) and 1 μl of 1:3 diluted RNA extracted via method A were applied to an RNA nanochip. (C) Separation of 30 μg protein extracted using the three methods in a 12% SDS–polyacrylamide gel, followed by Coomassie Brilliant Blue G250 staining. Each lane was cut into four parts and subjected to tryptic digestion followed by LC–MS/MS.

The resulting quantities of extraction for the different molecule classes are summarized in Table 1. The gDNA content determined via Nanodrop for method A cannot be considered as accurate because both RNA and DNA were present in the sample. In addition, the DNA obtained by method B was difficult to dissolve and remained viscous, making the determined quantity questionable. Nevertheless, the volume of DNA isolated with the tested methods was more than sufficient for standard genomic applications such as PCR and DNA sequencing, which need approximately 60 ng and 1 μg of gDNA, respectively.

RNA isolation: enrichment strategies, integrity, and yield

When using method A, the RNA was mixed with DNA and partially degraded, as shown by the 1.8% agarose gel. For methods B, C, and GS, no RNA degradation was detected (data not shown), and Bioanalyzer measurements confirmed its integrity, with RIN values ranging from 8.4 to 10, whereas values were 3.3–3.8 for method A (Fig. 2B). For downstream transcriptomic analyses, including qPCR applications, it is generally recommended to use RNA samples with a minimum RIN of 7 [14]. Methods B, C, and GS are suitable for the extraction of high-quality RNA that can be used in downstream applications.

We isolated approximately 28.7/79.6 μg (Jurkat/Hepa) of RNA from 1 × 10^7 cells using method A (Nanodrop/containing DNA), 78.9/380.7 μg using method B, 13.8/161.6 μg using method C, and 14.4/7.7 μg with GS (Table 1).

From Jurkat cells, we isolated approximately 28.7 μg of RNA using method A (Nanodrop), 78.9 μg using method B, 13.8 μg using method C, and 14.4 μg with GS. Hepa cells yielded approximately 79.6 μg with method A, 380.7 μg with method B, 161.6 μg with method C, and 7.7 μg with GS (Table 1).

Methods B, C, and GS yielded a quantity of RNA sufficient for further analyses such as RNA sequencing (requiring 500 ng of DNase-treated RNA) and reverse transcription followed by qPCR (recommended 5 μg of DNase-treated RNA). With method A, RNA and DNA were combined.

Protein isolation: enrichment strategies, purity, physicochemical bias, and yield

As with RNA and DNA, the yield of extracted proteins varied for the three protocols and the well-established method (GS). From Jurkat cells, method A produced 147.7 μg, method B 65.3 μg, method C 125.8 μg, and the well-established method 519.3 μg. Yield from Hepa cells was 465.4 μg for method A, 168.2 μg for method B, 323.2 μg for method C, and 961.3 μg for the well-established method (Table 1).

Resuspension of proteins using 1% SDS (method B) was difficult or impossible, and a large portion of the pellet remained undissolved. This was also reported by Simões and coworkers [15], who found that further modifications of the protocol, including urea/SDS solubilization and sonication, increased protein yield.

An equal volume of protein (30 μg) extracted by the three methods and GS were precipitated, resuspended in sample buffer, and separated in a 12% SDS–polyacrylamide gel (Fig. 2C). The peptides obtained by in-gel digestion were extracted and analyzed using LC–MS/MS. Coomassie-stained SDS–polyacrylamide gel indicated low molecular weight proteins (<35 kDa) to be underrepresented using method A compared with extraction via methods B and C (Fig. 2C). The tendency of proteins less than 25 kDa to be underrepresented in global proteome studies can be associated with factors such as lower efficacy of precipitation [16], loss during destaining [17], and a scarcity of readily detectable tryptic peptides [18].

Visual comparison of band patterns in SDS–gel lanes corresponding to extracts derived from methods A, B, and C revealed distinct similarities. High molecular weight proteins showed similar abundance in extracts of the three methods. For more comprehensive insight into the proteome data, the proteins/

Table 1
Yield, quality, and sensitivity of detection of the four classes of biomolecules extracted from 1×10^7 Jurkat T cells and Hepa 1c1c7 cells by the three methods.

Jurkat E6-1		GS	Method A	Method B	Method C
DNA	µg isolated	9.9	35.8	174.9	18.8
	quality	***	*	*	***
RNA	µg isolated	14.4	28.7	78.9	13.8
	quality	***	*	***	***
Protein	µg isolated	519.3	147.7	65.3	125.8
	quality	***	***	***	***
Metabolite	µg isolated	30/29	35	8	37
	quality	***	**	*	***

Hepa 1c1c7		GS	Method A	Method B	Method C
DNA	µg isolated	17.1	99.5	153.6	45.2
	quality	***	*	*	***
RNA	µg isolated	7.7	79.6	380.7	161.6
	quality	***	*	***	***
Protein	µg isolated	961.3	465.4	168.2	323.2
	quality	***	***	***	***
Metabolite	µg isolated	31/30	23	8	37
	quality	***	**	*	***

peptides identified in Jurkat T cells and Hepa cells by methods A, B, and C were analyzed in greater detail (Fig. 3). The detection overlap of the three methods was greater for Hepa cells than for Jurkat T cells (Fig. 3A). Mass spectrum analysis yielded similar numbers of identified proteins and demonstrated a broad overlap of the three protocols and the GS in Jurkat T cells (method A: 1091 identified proteins; method B: 1271; method C: 870) (Fig. 3C). The three methods show an overlap of 792 identifications. Methods A and B show an overlap of 23 + 792 hits, methods B and C show an overlap of 34 + 792 , and methods A and C show an overlap of 231 + 792. In addition, 35 unique proteins were extracted and identified using method A, 221 using method B, and 21 using method C.

Numbers and overlap of identified proteins using pellets of Hepa 1c1c7 cells are also shown in Fig. 2.

A critical factor in extraction protocols is systematic bias due to physicochemical properties of the target molecules. This is not supposed to be crucial for molecules like RNA and DNA whose building blocks are rather homogeneous but highly relevant when the molecules differ substantially, as is the case with proteins. Therefore, we analyzed the physicochemical parameters of the detected proteins from Jurkat and Hepa cells: hydrophobicity (by GRAVY [grand average of hydropathy] score), isoelectric point (p*I*), and molecular weight (MW) (Fig. 3B). The GRAVY scores calculated according to Kyte and Doolittle [19] showed a Gaussian-type distribution pattern in the range of −2 to approximately +0.5 with a maximum of −0.5 and a frequency of 120–160. The pattern showed high similarity of extracted proteins to the GRAVY distribution of the annotated human proteome (UniProt 09/2013), but the GRAVY range of +0.2 to +1 was underrepresented, indicating that strongly hydrophobic proteins were not readily targeted using the three methods. For strongly hydrophobic proteins, we recommend the use of dedicated protocols. Another possible reason for the relatively low recovery of hydrophobic proteins might be the precipitation approach used. A study of urine samples of limited complexity revealed that hydrophobic precipitation favored the enrichment of acidic and hydrophilic proteins and showed a negative bias against hydrophobic proteins [20]. Because the methods compared here used hydrophobic precipitation, it is not surprising that they show a similar bias. There were differences in the proteomic profiles obtained by the three methods; hence, the comparison of proteomic analyses based on different extraction protocols is problematic.

The p*I* profiles of identified proteins showed a similar distribution across extraction methods. They displayed two well-separated peaks: one at p*I* 4.0 to 7.0, with a maximum of 5.0 and a frequency of 140, and a second at p*I* 7.0 to 12.0, with a maximum of 9.0 and a frequency of 80. Compared with the p*I* distribution of the annotated human proteome, the p*I* 8 to 10 protein fraction was underrepresented in the protein samples extracted via methods A, B, and C.

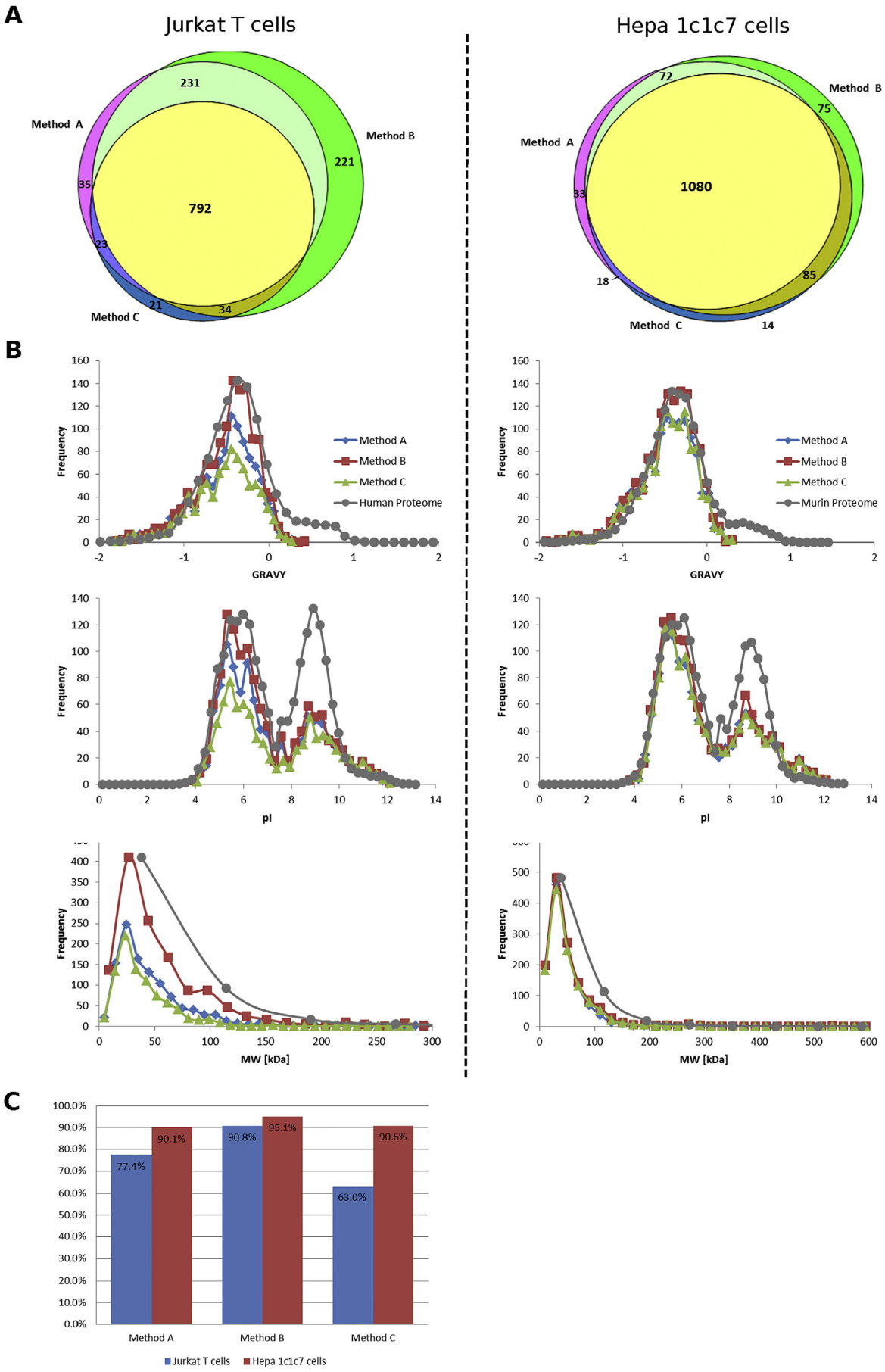

Fig.3. Overlap and physicochemical properties of identified proteins. Proteins were identified by LC–MS/MS after tryptic digestion of 30 μg protein extracted by the three methods from 1×10^7 Jurkat T cells and 1×10^7 Hepa 1c1c7 cells. (A) Number and overlap of proteins identified. (B) GRAVY, MW, and p*I* distribution of the corresponding proteome (human or murine) and of identified proteins. (C) Proportion of identified proteins isolated by the "gold standard" protein extraction method that was also found with methods A, B, and C.

The identified proteins showed a similar MW distribution across methods, with a maximum frequency of 350–400 at 35–40 kDa and a gradual decrease in the range of 75–200 kDa, with few proteins detected above 200 kDa. The three MW distribution profiles correlated well with the hypothetical distribution profile of the annotated human proteome.

Metabolite extraction: enrichment strategies, purity, and yield

As a benchmark for the efficacy of metabolite extraction, we used an established targeted multianalyte (>40) approach [13] because we were primarily interested in the primary products of carbon and nitrogen metabolism. Method B was not suitable for the extraction of metabolites by IC–MS/MS analysis because the detergents of the TRI Reagent used have a negative influence on the detectability of target analytes. In the analysis of samples from method B, the MRM chromatograms were characterized by a high level of background noise and a longer reequilibration time for consecutive runs.

Method A produced 35 detectable metabolites in Jurkat cells and 23 in Hepa cells, method C produced 37 in both cell types, GS1 produced 30 in Jurkat cells and 31 in Hepa cells, and GS2 produced 29 in Jurkat cells and 30 in Hepa cells (Fig. 4). All metabolites detected with method A were present in the MRM chromatograms of method C. The intensities were higher in method C by a factor of 5–15, indicating clearly higher recovery of the target analytes by

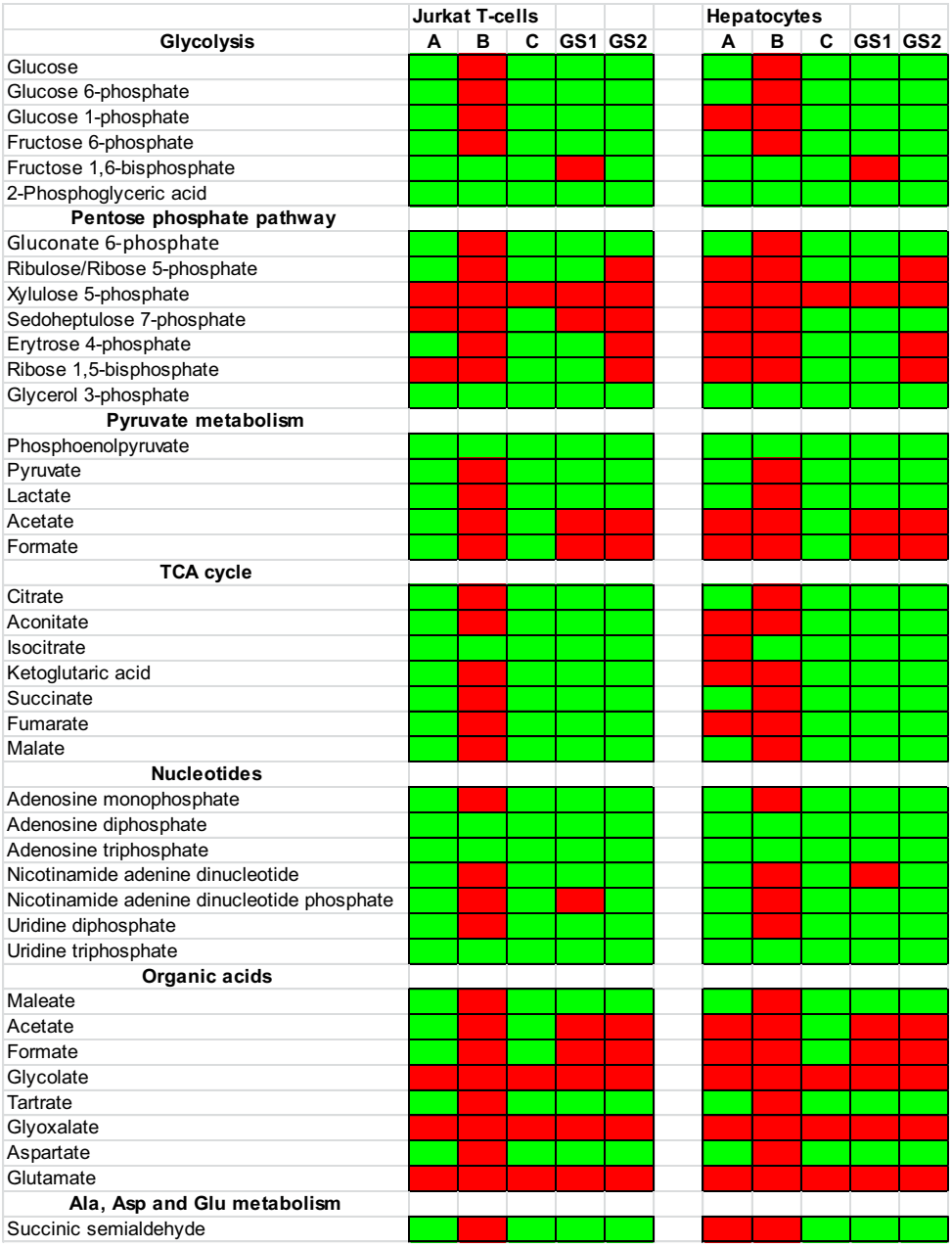

Fig.4. Metabolites determined by IC–MS/MS extracted by the three methods. Green boxes indicate potential quantitative measurements, and red boxes indicate no metabolite detection. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

method A. At least six metabolites detected with the identical instrumental setup in earlier studies [12,21] were not found after extraction method A in this study.

Conclusions

The comparison of three methods for simultaneous extraction of DNA, RNA, proteins, and metabolites from Jurkat T cells and hepatocytes showed adequate agreement between the cell lines. Method A was found to be suitable for metabolite and protein extraction, but not for specific extraction of RNA and DNA, and produced slightly lower detection of metabolites than method C. Method B provided high-quality RNA and protein samples but nearly unresolvable DNA and protein. Our attempts to add metabolite extraction to method B failed, as assessed by IC–MS/MS. Only method C provided DNA, RNA, protein, and metabolite samples that passed all quality benchmarks in both tested cell lines.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.05.011.

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